

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
3 May 2001 (03.05.2001)

PCT

(10) International Publication Number
WO 01/31048 A1

- (51) International Patent Classification⁷: C12P 13/00, A61K 35/78 (74) Agent: LEE, Kuiy, Dong; Seoul Building, 114-31, Urdong, Chongro-ku, Seoul 110-350 (KR).
- (21) International Application Number: PCT/KR00/01227 (81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (22) International Filing Date: 27 October 2000 (27.10.2000)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 1999/46913 27 October 1999 (27.10.1999) KR (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
- (71) Applicant (*for all designated States except US*): KOREA FOOD RESEARCH INSTITUTE [KR/KR]; San 46-1, Backhyun-dong, Bundang-ku, Sungnam, Kyungki-do 463-420 (KR).
- (72) Inventors; and (75) Inventors/Applicants (*for US only*): RHYU, Mee, Ra [KR/KR]; Sambu Apartment 408-1502, Saeppyul-maul, Bundang-dong, Bundang-ku, Sungnam, Kyungki-do 463-030 (KR). KIM, Duk, Kyung [KR/KR]; Family Apartment 227-706, Munjeong-dong, Songpa-ku, Seoul 138-200 (KR). KIM, Bo, Kyung [KR/KR]; Yeonsu-jugong Apartment 406-1503, Yeonsu-dong, Chungju, Chungcheongbuk-do 380-100 (KR).

Published:

- With international search report.
- Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NITRIC OXIDE DONOR COMPOSITION USING RED RICE FERMENTED WITH *MONASCUS* AND A PHARMACEUTICAL COMPOSITION CONTAINING THE SAME

(57) Abstract: The present invention is for a nitric oxide donor composition and a pharmaceutical formulation containing the same. The nitric oxide donor composition is prepared by using red rice fermented with *Monascus* fungus, its powder or aqueous extract form or combinations thereof. Compositions according to the present invention exhibit vasorelaxing effects without causing side effects harmful to the vascular system.

EXPRESS MAIL NO.:

EV327549117US

WO 01/31048 A1

**NITRIC OXIDE DONOR COMPOSITION USING RED RICE FERMENTED
WITH *MONASCUS* AND A PHARMACEUTICAL COMPOSITION
CONTAINING THE SAME**

5

FIELD OF THE INVENTION

The present invention is directed to a nitric oxide donor composition and a pharmaceutical preparation containing the same. Specifically, the present invention is directed to a composition comprising red rice fermented with *Monascus* fungus, the powder form thereof or the aqueous extract, as an active ingredient in a pharmaceutical formulation.

15

BACKGROUND OF THE INVENTION

In general, vascular smooth muscle control blood motility by alternating between relaxation and constriction. Blood vessels of patients with diseases in the circulatory system, such as hypertension, hypercholesterolemia, etc., fail to properly relax and constrict. Such malfunction of the blood vessels is associated with nitric oxide ("NO") which is released from vascular endothelium. Vascular endothelium plays an important role in homeostasis, such as control of blood flow, blood vessel tension and suppression of thrombocyte coagulation. Once vascular endothelium is damaged, NO release from endothelium decreases, which in turn prevents normal relaxation and constriction of blood vessels.

25

The blood vessel relaxation mechanism has been widely known. The mechanism is summarized as follows. Vascular endothelium releases NO, an endothelium-derived relaxing factor, and the released NO acts on vascular smooth muscles to activate soluble guanylate cyclase. The activated guanylate cyclase stimulates increase in cyclic GMP levels. The increased cyclic GMP relaxes blood vessels.

30

Studies on NO have been rapidly developed since Furchgott and Zawadzki's research in 1980 (Nature, 288:373, 1980). The basic mechanism of NO biosynthesis

was discovered in the earlier part of the 1990's. Recently, NO studies have been focused on the pathology and the treatment of related diseases, which is now one of the most interesting research areas in medicine.

5 Thus, NO studies on the vascular circulatory system are known. Currently, NO studies are applied for all of medical fields such as the autonomic nervous system, endocrine system, central nervous system, immunology, etc. Clinically, it was reported 120 years or more ago that nitroglycerin is effective as a therapeutic agent for angina. Since then, NO producing material (NO donor) have been used as a
10 therapeutic agent for heart disease even though the mechanism of NO's action was not clear.

 Further, NO or NO-related material has a role in alleviating essential hypertension, pulmonary hypertension and renal hypertension, and is an essential
15 material for treating human vessel diseases (ischemic heart disease). Examples of diseases for which NO donors are used as a therapeutic agent include angina, myocardial infarction, hypertension, etc. The NO donor may be administered as an injection, sublingual agent, patch, spray, inhalant, etc. In addition, NO is related with impotence in males. In this regard, VIAGRA® from Pfizer Inc., which has
20 recently been highlighted, is directly associated with NO.

 It is clear that the scope of diseases for which NO or NO-related materials may be used as a therapeutic agent will become wider in the future. Such an NO donor may be used as a therapeutic agent, i.e. vasorelaxing agent for preventing
25 and/or treating diseases in the circulatory system such as hypertension, cerebral hemorrhage, arteriosclerosis, disorders in hemocirculation, neuralgia, diabetes, sexual dysfunction, memory impairment, and the like. Thus, numerous pharmaceutical companies throughout the world have attempted to develop an efficient vasorelaxing agent, and many commercially available agents, including VIAGRA®, have been
30 produced.

 Vasorelaxing agents (NO donors) are classified into chemical drugs and crude drugs (i.e. medicinal herbs). First, conventional chemical drugs frequently cause undesirable side effects such as damage to vascular endothelium, hemolysis,

acute or subacute toxicity, acute nephritis and cardioplegia. Further, their production processes are very complex, and mass production is difficult. It has been especially reported that some chemical vasorelaxing agents including VIAGRA® cause side effects that is pernicious to life such as cardioplegia. Thus, presently, chemical
5 vasorelaxing agents may not be safe therapeutic agents.

In comparison with chemical drugs, most identified crude drugs do not cause pernicious side effects as they have been in use for quite some time, indicating that they have been clinically tested. For example, crude drugs (vasorelaxing agents)
10 which contain gensenosides Rg₁ and Rg₂ as major components (Korean Patent Publication No. 1999-201585), no side effect occur.

As presented above, crude drugs may have less side effects than chemical drugs. However, it may be culturally or physically difficult to obtain source
15 materials for crude drugs such as wild ginseng, cultivated ginseng, rhinoceros horn, tiger bone, bear gall bladders, and other rare animals or plants. Even if such source materials are available, they are generally high-priced articles. Furthermore, in most cases, the source materials contain a very small amount of an effective component necessary to produce a reliable vasorelaxing agent.

20 Therefore, there is a continuing need for a novel vasorelaxing agent (NO donor) which can be used for preventing and/or treating diseases such as angina, myocardial infarction, heart disease, hypertension, cerebral hemorrhage, arteriosclerosis, disorders in hemocirculation, neuralgia, diabetes, sexual dysfunction,
25 memory impairment and the like, which does not cause side effects and have low production costs.

SUMMARY OF THE INVENTION

30 The present invention provides a nitric oxide donor composition and a pharmaceutical preparation containing the same. Specifically, the present invention provides a nitric oxide donor composition comprising red rice fermented with *Monascus* fungus and/or powder of said red rice and/or extract of said red rice, as an active compound.

The present invention also provides a nitric oxide donor composition which may be used for manufacturing a vasorelaxant that has no side effects, and through a simple process with low production costs.

5

The present invention also provides a nitric oxide donor composition which may be used for manufacturing a therapeutic agent for treating hypertension, impotence, myocardial infarction, angina or memory impairment through a simple process with low production costs.

10

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a graph illustrating endothelium-dependency of the composition of the present invention.

15

Fig. 2 is a graph illustrating concentration-dependency of the composition of the present invention.

20

Fig. 3 is a graph illustrating effects of a nitric oxide inhibitor on the composition of the present invention.

Fig. 4 is a graph illustrating effects of GABA and atropine on the vasorelaxation induced by the composition of the present invention.

25

Fig. 5 is graph illustrating a comparison of the amounts of nitric oxide produced in vascular endothelium in the presence of the composition of the present invention and L-NNA, histamine.

DETAILED DESCRIPTION OF THE INVENTION

30

This description discloses the discovery that red rice fermented with *Monascus* fungus the powder of such red rice and extracts of such red rice can act as NO donors, i.e. a vasorelaxant.

Thus, the present invention provides a nitric oxide donor composition and a pharmaceutical formulation containing the same. The nitric oxide donor composition according to the present invention comprises, individually or in combination, red rice fermented with *Monascus*, the powder of such red rice, and
5 extracts from such red rice as an active compound. Thus, the present invention provides a new use of red rice fermented with *Monascus* and its derivatives as an active ingredient.

In one aspect of the present invention, a nitric oxide donor composition may
10 comprise milled powder of red rice fermented with *Monascus*. The fermented red rice is prepared by using red rice as a source material and a *Monascus* species, which are readily available to one of ordinary skill the art as a microorganism used for fermentation. For example, the fermented red rice may be prepared by a process comprising the steps of:

- 15 (a) soaking milled rice for 3 hours in water at 50 °C;
- (b) draining, cooking by autoclave and cooling the soaked rice; and
- (c) inoculating the cooled rice with *Monascus ruber* IFO32318 and fermenting for 2 days at 30 °C and subsequently for 6 days at 25 °C under aerobic conditions.

20

In another example, the powder of the fermented red rice may be prepared by a process comprising the steps of:

- (a) soaking milled rice for 3 hours in water at 50 °C;
- (b) draining, cooking by autoclave and cooling the soaked rice;
- 25 (c) inoculating the cooled rice with *Monascus ruber* IFO32318 and fermenting for 2 days at 30 °C and subsequently for 6 days at 25 °C under aerobic conditions;
- (d) heating the fermented rice at 90 °C for 20 min to inactivate the fungal enzymes;
- 30 (e) drying the heated rice at 50 °C to lower than 10% moisture content; and
- (f) grinding the dried rice in a commercial mixer.

In another aspect of the present invention, a nitric oxide donor composition may comprise an extract of red rice fermented with *Monascus*. The extract may be

obtained by any extraction method which is conventionally used in the art. For example, the extract of the fermented red rice may be prepared by a process comprising the steps of:

- 5 (a) immersing fermented red rice in 4 vols of ethanol at 80 °C for 1 hour, repeat three times;
- (b) condensing the extracts obtained in step (a) and making a suspension in water;
- (c) mixing the suspension with ethyl acetate;
- 10 (d) washing the aqueous phase with ethyl acetate and subsequently with butanol;
- (e) evaporating the aqueous phase; and
- (f) dissolving the solids in saline.

15 When red rice fermented with *Monascus* or its powder is used, the content of the active compound in the red rice or the powder may be small, and its standardization may be difficult. Thus, when red rice fermented with *Monascus* or its powder is used clinically for prevention or treatment of diseases, the amount of active compound to be used needs to be adjusted. It is essential to standardize the content of the active compound with a suitable control method, especially for a mass
20 production of pharmaceuticals.

The NO donor composition according to the present invention as described above can be mixed with a conventional pharmaceutical carrier to produce conventional pharmaceutical formulations. For example, an oral formulation such as
25 tablets, capsules, troches, suspensions and sublinguals; an injectable formulation such as an IV or IM solution or suspension; a dried powder that can be instantly used with distilled water for injection; and a topical formulation such as ointment, cream, patches, liquid lotion, spray or inhalant.

30 Suitable carriers that can be used together with "WP/FRM" (water phase of fermented rice with *Monascus*) of the present invention, include for oral formulation, binders, lubricants, disintegrators, diluents, solubilizers, dispersing agents, stabilizers, emulsifiers, pigments and flavors. For injection formulations, preservatives, pain relieving agents, solubilizers or stabilizers can be used. For a topical formulation,

bases, diluents, lubricants or preservatives can be used.

Pharmaceutical formulations prepared as described above can be administered via oral or non-oral routes, e.g. intravenous, subcutaneous or intraperitoneal routes. As mentioned previously, it can be tropically administered. Additionally, in case of oral administration, to prevent the formulation from being degraded by gastric acids, an antacid can be used in combination. As an alternative, solid formulations such as tablet can be subjected to enteric coating.

A carrier and an NO donor composition of the present invention can be mixed and compressed to form tablets. They can also be granulated and filled to produce capsules. Also, they can be produced as an injection formulation by addition of distilled water for injection and glycerin, or can be produced as a wet pack or patch type formulation.

The NO donor composition of the present invention as described above can be produced from ordinary rice and microorganisms such *Monascus* as main raw materials, thus production cost is very low compared to conventional pharmaceuticals or traditional medicines dependent on a rare plant such as ginseng. Further, the production procedure comprises fermentation, pulverization and extraction, which are simple production processes which are suitable for mass production. In addition, the NO donor of the present invention exhibits a remarkable relaxing effect on blood vessels without serious negative side effects such as hemolysis and toxicity, while not causing damage to blood vessel endothelium. Thus, it exhibits very stable physiological activity and safety in use.

Further, the NO donor composition may be used for manufacture therapeutic agents for preventing and/or treating diseases that are caused by constrictions in the circulatory system, such as angina, myocardial infarction, heart diseases, hypertension, cerebral hemorrhage, arteriosclerosis, disorder in hemocirculation, neuralgia, diabetes, sexual dysfunction, memory impairment, etc.

As will be explained in detail below, the NO donor composition of the present invention induces endothelium-dependent and concentration-dependent blood

vessel relaxation. The blood vessel relaxation occurs because the NO donor composition of the present invention directly stimulates endothelium to produce NO. This is the same with the red rice fermented with *Monascus* or its powder or extracts

- 5 The sections below will describe whether and how NO donor compositions of the present invention, comprising red rice fermented with *Monascus*, powdered red rice or extracts of the red rice can be used for manufacture of vasorelaxants. For simplicity, the present invention will be described with reference to extracts of the red rice as this is equally applicable to the fermented red rice or the powdered rice.
- 10 Additionally, the specific procedures and methods described herein are merely illustrative for the practice of the present invention and are by no means intended to limit the scope thereof. Analogous procedures and techniques are equally applicable as will be clear to ones skilled in the art after having the benefit of this disclosure.

15

EXAMPLE 1

PREPARATION OF RED RICE FERMENTED WITH *MONASCUS*, POWDERD RED RICE AND EXTRACT OF THE RED RICE

- 20 Milled rice was soaked for 3 hours in water at 50 °C, drained and cooked by autoclave. It was then inoculated with *Monascus ruber* IFO32318 and fermented for 2 days at 30 °C and subsequently for 6 days at 25 °C under aerobic conditions. The fermented rice was heated at 90 °C for 20 min to inactivate the fungal enzymes and dried at 50 °C to lower than 10% moisture content and ground with a commercial
- 25 mixer.

- Red fermented rice was extracted using the method described by Kohama Y. et al. (Chemical and Pharmaceutical Bulletin, 35:2484-2489, 1987). In brief, red rice (1 kg) was immersed three times in 4 vols of ethanol at 80 °C for 1 hour and the
- 30 extracts (5.21g) were evaporated and suspended in water, before the suspension was mixed with ethyl acetate (AcOEt). The water phase was first washed with AcOEt and then with butanol (BuOH). The water phase was evaporated and the solids (1.54 g) dissolved in saline. As noted previously, the resulting water phase of fermented rice with *Monascus* is called 'WP/FRM' (water phase fermented rice with *Monascus*).

A specimen (No. HG-0001) has been deposited with the Korea Food Research Institute (KFRI), Kyungki-Do, South Korea.

EXAMPLE 2

5

VASORELAXING ACTIVITY OF WP/FRM AND THE ENDOTHELIUM-DEPENDENCY THEREOF

10 This example was designed to examine whether WP/FRM exhibits
vasorelaxing activity and whether the vasorelaxing activity is endothelium-dependent.

Male Sprague-Dawley rats (200-250 g) were stunned and bled. The thoracic aorta was isolated and cut into rings (2-3 mm wide). In some experiments, the endothelium was removed by gently rubbing the inner surface of the vessel with
15 cotton thread moistened with physiological salt solution (PSS). Each strip of aorta was attached to a holder under a resting tension of 10 mN. After equilibration for 20 min in a 4-ml horizontal-type muscle bath, each strip was repeatedly exposed to 70mM KCl solution until responses became stable. The PSS contained (mM): NaCl 136.9; KCl 5.4; CaCl₂ 1.5; MgCl₂ 1.0; NaHCO₃ 23.8; glucose 5.5; and
20 ethylenediaminetetraacetic acid (EDTA) 0.01. The high K⁺ solution was prepared by replacing NaCl with equimolar KCl. These solutions were saturated with a 95% O₂ and 5% CO₂ mixture at 37°C and pH 7.4. Muscle tension was recorded isometrically with a force-displacement transducer (FT03, Grass, RI, USA) connected to a polygraph system (RPS212, Grass, RI, USA) and a computer analyser (Power
25 Lab 400, MacLab system, Castle Hill, Australia).

The functional activity of vascular endothelium was assessed by measuring whether 1 μ M carbachol induced almost complete relaxation (> 90%) in aorta stimulated with 300 nM norepinephrine ('NE') (Sudjarwo et al., European Journal of
30 Pharmacology, 229:137-142, 1992). Subsequently, the aorta samples were treated with NE (300 nM) to stimulate vasoconstriction and then the relaxants (WP/FRM, carbachol or GABA) were added to the bath for 20min. L-NNA, atropine or indomethacin was used in pretreatment for 30 min before tensions were stimulated by NE. The relaxation induced by drugs was expressed as a percentage of the NE-

- 10 -

induced tension.

The results of Example 2 are shown in Fig. 1. Fig 1a is when vascular endothelium is present and Fig 1b is when vascular endothelium is absent. As can be seen in Figs. 1A and 1B, WP/FRM exhibits a vasorelaxing effect only when vascular endothelium is present. These experimental result mean that WP/FRM of the present invention shows its vasorelaxing effect without damaging vascular endothelium. Thus, it is evident that WP/FRM can be safely used as a vasorelaxant without side effects such as hemolysis, acute or subacute toxicity, etc.

More specifically, in the quiescent preparation, WP/FRM (0.1-10mg/ml) did not evoke any tension in the isolated rat aorta with or without endothelium (n=4, data not shown). Fig. 1A shows a typical trace of the effect of WP/FRM on muscle tension stimulated with 300 nM NE in endothelium-intact rat aorta. WP/FRM (1 mg/ml) relaxed transiently the endothelium-intact aortic preparation which was contracted with NE. The inhibited tension by WP/FRM gradually recovered to about 60% of the tension level before stimulation within 40 min after reaching the maximum relaxant response. In contrast, WP/FRM, 1 mg/ml, did not affect the tension stimulated by 300 nM NE in the endothelium-denuded aorta (Fig. 1B). The inhibitory effect of WP/FRM was similar to that brought about by 1 μ M carbachol, which induced over 90% relaxation when applied after an NE-induced tension ($97.0 \pm 3.93\%$).

EXAMPLE 3

CONCENTRATION-DEPENDENCY OF WP/FRM'S VASORELAXING ACTIVITY

This example was designed to examine whether WP/FRM exhibits a vasorelaxing activity in a concentration-dependent manner.

WP/FRM's of 0.1, 0.3, 1 and 3 mg/ml were presented to blood vessels with endothelium-intact. The averaged magnitudes of the relaxation response to WP/FRM are summarized in Fig. 2. Fig. 2 shows that relaxation response to

WP/FRM of 1.0 mg/ml or more is high, while relaxation response to WP/FRM of 0.3 mg/ml or less is low. From these results, it can be seen that the relaxing effect of WP/FRM increased in a concentration-dependent manner.

5

EXAMPLE 4

EFFECTS OF NITRIC OXIDE SYNTHESIS INHIBITOR ON WP/FRM-INDUCED RELAXATION

10

This example was designed to examine whether WP/FRM-induced vasorelaxation is brought on by NO produced in endothelium. To this end, the effects of L-NNA (an inhibitor of NO synthase) and indomethacin (a cyclooxygenase inhibitor), on relaxation induced by carbachol or WP/FRM (3 mg/ml) were examined.

15

Blood vessel samples were treated with L-NNA (10 μ M) for 30 min and then treated with NE (300 nM). To these samples, WP/FRM and carbachol (known as inducing endothelium-dependent vasorelaxation) were added. In addition, separate blood vessel samples were treated with indomethacin (10 μ M) for 60 min and then treated with NE (300 nM). To these samples, WP/FRM were introduced.

20

The results of these experiments are shown in Fig. 3. Fig. 3A represents results of experiments with WP/FRM treatment and with L-NNA pretreatment. Fig. 3B represents a comparison between experimental results of WP/FRM treatment with L-NNA pretreatment and those of WP/FRM treatment without L-NNA. Fig. 3 shows that when blood vessel samples treated with L-NNA and subsequently treated with NE were treated with WP/FRM, no relaxation was observed. In the carbachol treatment experiment, no relaxation was observed either (data not shown). However, when blood vessel samples treated with indomethacin and subsequently treated with NE were treated with WP/FRM, strong relaxation was observed (data not shown). From these results, it can be seen that WP/FRM induced a NO-mediated relaxation. This is uniquely and first described by the present disclosure.

30

EXAMPLE 5

EFFECTS OF GABA OR ACH ON THE WP/FRM-INDUCED RELAXATION

5 This example was designed to examine which of GABA (gamma-aminobutyric acid) or Ach (acetylcholine), which are known hypotensive compounds, found in the aqueous fraction of red rice mold fermented with *Monascus pilosus* (Kohama et. al. Chemical and Pharmaceutical Bulletin, 35:2484-2489, 1987), is responsible for the relaxation effect.

10

Blood vessel samples were treated with atropine, a muscarinic receptor antagonist (1 μ M) and then treated with NE (300 nM). WP/FRM were treated to these samples (Fig. 4A). In addition, separate blood vessel samples were treated with NE (300 nM). To these samples, GABA was further applied (Fig. 4B).

15

The results of these experiments are shown in Fig. 4. The WP/FRM-induced relaxation was not significantly different in the presence or absence of 1 μ M atropine (Fig. 4A). The maximal relaxation induced by 3 mg/ml of WP/FRM were $93.0 \pm 3.25\%$ (Fig. 2) and $87.9 \pm 6.21\%$ in the presence and absence of atropine, respectively. But the recovery of WP/FRM-induced relaxation was faster in the presence of atropine than that in the absence of atropine (Fig. 1A vs. Fig. 4A). This indicates that Ach is partly responsible for NO-mediated endothelium-dependent relaxation induced by WP/FRM. In addition, GABA (350 nM) did not affect the increase in tension induced by NE in endothelium-intact aorta (Fig. 4B).

25

EXAMPLE 6

NITRIC OXIDE PRODUCTION BY WP/FRM

30 The present example was designed to examine whether nitric oxide production in endothelium is directly stimulated by WP/FRM.

1. CELL CULTURE

Endothelial cells were enzymatically isolated from human umbilical cord vein. The enzyme mixture contained 0.2% collagenase type II in 0.2% glucose-phosphate-buffered saline (PBS). Human umbilical cord vein was washed with 0.2% glucose-PBS, then filled with 10 ml of the enzyme mixture. The umbilical cord was incubated at 37°C for 5 to 7 min. The collagenous effluent was collected and inactivated with fetal calf serum to a final concentration of 30%. The vein was washed with 50 ml of 0.2% glucose-PBS. The pooled effluents containing the freed endothelial cells were centrifuged at 1000 rpm for 5 min.

The cell pellet was dispersed in growth medium that contained Medium 199, fetal calf serum 20%, penicillin streptomycin (100 U/ml and 100 µg/ml), endothelial cell growth supplement (ECGS) 50 µg/ml, and heparin 15 U/ml. Cells were plated into T25 cm² flasks and incubated at 37°C in a humidified atmosphere of 5% CO₂. The cells were grown to confluence, detached by incubation in PBS containing 0.01% trypsin and 0.004% EDTA for 1-2 min at room temperature, washed with the culture medium, then centrifuged and re-seeded onto 24-well plates. Cells between the first and second passages were used.

2. MEASUREMENT OF NITRIC OXIDE RELEASE

The endothelial cells grown to confluence were washed three times with Krebs-Hepes buffer (mM): NaCl 99.0, KCl 4.69, CaCl₂ 1.87, MgSO₄ 1.2, NaHCO₃ 25, K₂HPO₄ 1.03, Hepes 20, D-glucose 11.1 (pH 7.4). Either WP/FRM or histamine dissolved in Krebs-Hepes buffer were added to the wells and the cells were incubated at 37°C for 10 min. Cells were pretreated with L-NNA for 10 min. After incubation, the supernatant was collected from each well and centrifuged at 12,000 rpm for 5 min to remove cells. The production of NO was measured by combining nitrite (NO₂⁻) and nitrate (NO₃⁻) using the NO assay kit according to the manufacturer's recommendations.

Briefly, nitrate was first enzymatically reduced to nitrite by incubating 50 µl of the sample with 25 µl NADPH and 25 µl of nitrate reductase for 30 min at 37°C.

- 14 -

For quantification of the nitrite concentration, 100 μl of reduced sample to be analyzed was mixed with 50 μl of sulphanilic acid containing HCl solution. After mixing for 10 min, 50 μl of N-(1-naphthyl)ethylenediamine solution was added to produce a diazo compound, which leads to a purple azo dye. The coloured azo dye
5 was measured 10 min later by enzyme-linked immunosorbent assay (ELISA) reader at 540 nm (Molecular Devices Corporation, Sunnyvale, CA, USA).

As described above, to demonstrate that WP/FRM directly stimulates vascular endothelium to release NO, the production of NO was measured in cultured
10 endothelial cells stimulated with WP/FRM. The measurement results are shown in Fig. 5. Stimulation of the primary cultured endothelium from human umbilical cord vein with WP/FRM (30 $\mu\text{g/ml}$) led to about double the content of nitrite and nitrate. The increased nitrite and nitrate content induced by WP/FRM was attenuated by the addition of L-NNA (10 μM) (Fig. 5). Similar results were obtained for nitrite and
15 nitrate production from the endothelium treated with histamine (10 μM).

From these results, it can be seen that WP/FRM directly affects NO production. This discovery is also uniquely and first disclosed herein.

* * * * *

20

The present invention has been described with reference to various specific examples. However, it should be understood that numerous variations and modifications are possible to those skilled in the art without departing from the spirit of the present invention, and all such variations and modifications are intended to be
25 within the scope of the claims which follow.

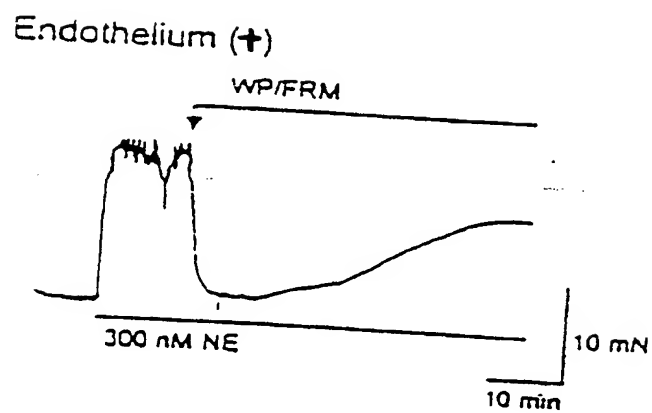
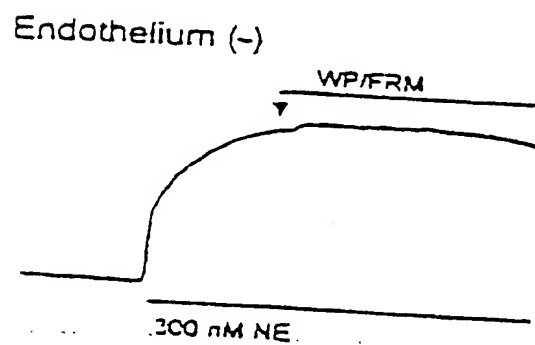
WHAT IS CLAIMED IS:

1. A nitric oxide donor composition comprising an active compound selected from the group consisting of red rice fermented with *Monascus*, a powder of red rice
5 fermented with *Monascus*, an extract of red rice fermented with *Monascus*, and combinations thereof.
2. A nitric oxide donor composition according to claim 1 wherein the fermentation is carried out with *Monascus ruber* IFO32318.
10
3. A nitric oxide donor composition according to claim 1 wherein the fermented red rice is prepared by a process comprising the steps of:
 - (a) soaking milled rice for 3 hours in water at 50 °C;
 - 15 (b) draining, cooking by autoclave and cooling the soaked rice; and
 - (c) inoculating the cooled rice with *Monascus ruber* IFO32318 and fermenting for 2 days at 30 °C and subsequently for 6 days at 25 °C under aerobic conditions.
- 20 4. A nitric oxide donor composition according to claim 1 wherein the powder of the fermented red rice is prepared by a process comprising the steps of:
 - (a) soaking milled rice for 3 hours in water at 50 °C;
 - (b) draining, cooking by autoclave and cooling the soaked rice;
 - 25 (c) inoculating the cooled rice with *Monascus ruber* IFO32318 and fermenting for 2 days at 30 °C and subsequently for 6 days at 25 °C under aerobic conditions;
 - (d) heating the fermented rice at 90 °C for 20 min to inactivate the fungal enzymes;
 - 30 (e) drying the heated rice at 50 °C to lower than 10% moisture content; and
 - (f) grinding the dried rice with a commercial mixer.
5. A nitric oxide donor composition according to claim 1 wherein the extract of the fermented red rice is prepared by a process comprising the steps of:

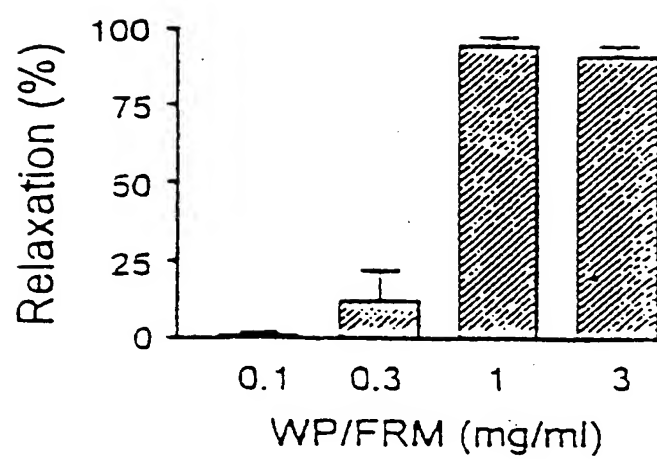
- 16 -

- 5 (a) immersing the powder of the fermented red rice in volumes of ethanol at 80 °C for 1 hour three times;
- (b) evaporating the extracts obtained in the step (a) and making a suspension in water;
- (c) mixing the suspension with ethyl acetate;
- (d) washing the water phase with ethyl acetate and subsequently with butanol;
- 10 (e) evaporating the water phase; and
- (f) dissolving the solids in saline.
6. A nitric oxide donor composition according to claim 1 which is characterized in that the composition is manufactured as a unit dosage form applicable for vasorelaxation with a pharmaceutically acceptable carrier.
- 15 7. A nitric oxide donor composition according to claim 1 wherein the amount of the active compound in the red rice, the powder, or the extract is 0.3mg/ml or more.
8. A nitric oxide donor composition of any one of claims 1 to 7 for the
- 20 manufacture of a vasorelaxant.
9. A nitric oxide donor composition of any one of claims 1 to 7 for the manufacture of a medical therapeutic agent for treating diseases comprising hypertension, impotence, myocardial infarction, angina, or memory impairment.

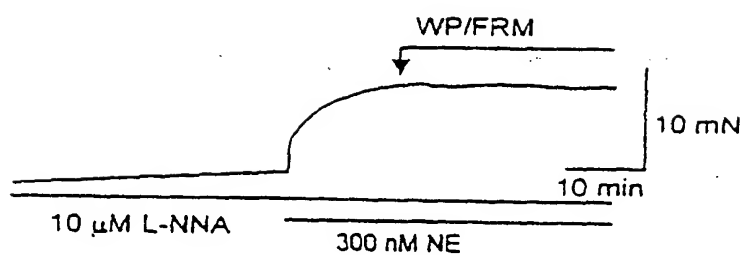
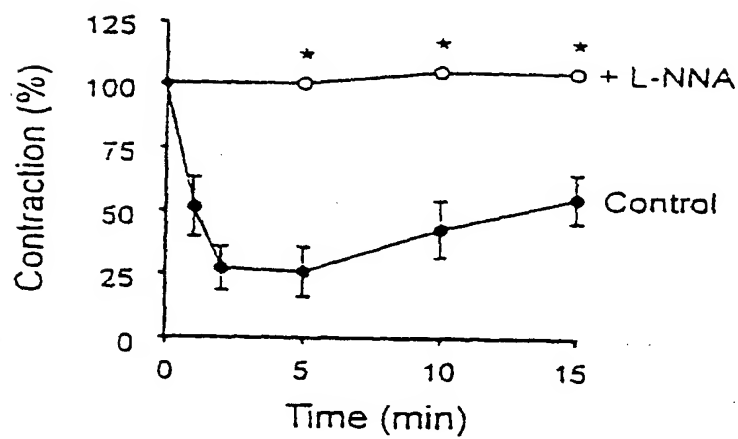
1/5

Fig. 1A*Fig. 1B*

2/5

Fig. 2

3/5

Fig. 3A*Fig. 3B*

4/5

Fig. 4A

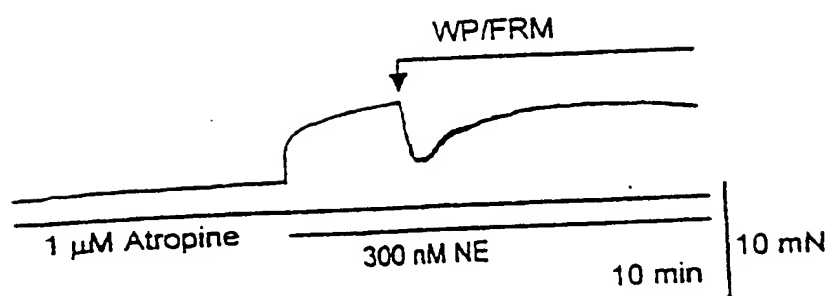
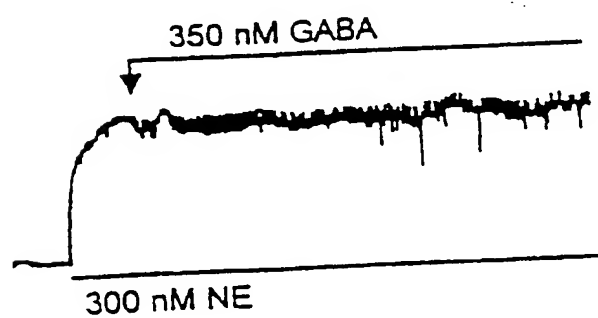
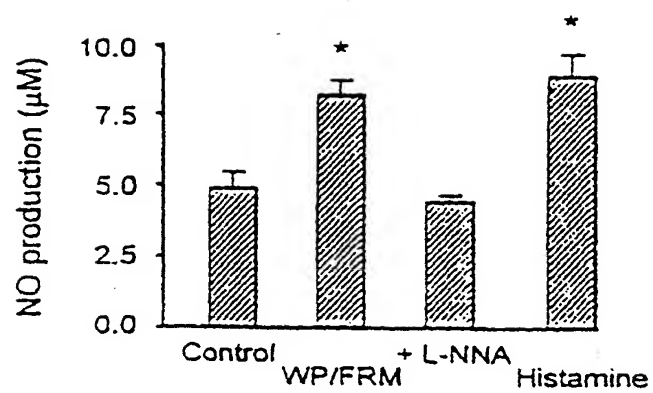


Fig. 4B



5/5

Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 00/01227

CLASSIFICATION OF SUBJECT MATTER

IPC⁷: C12P 13/00, A61K 35/78

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC⁷: C12P, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99/23996 A2 (PEKING UNIVERSITY) 20 May 1999 (20.05.99) abstract, claims 5, 6, 13-16	1-4, 8, 9
A	JP 5-056 774 A (NAGANO PREV GOV) 30 September 1993 (30.09.93) (abstract). [online] [retrieved on 10-1-2001]. Retrieved from: EPOQUE PAJ Database abstract	1-4
A	WO 98/42661 A1 (YSSUM RESEARCH DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM) 1 October 1998 (01.10.98) pages 2 and 3	9-11
A	WO 99/37616 A1 (ÄNGGARD et al.) 29 July 1999 (29.07.99) claims 1, 9-11	6-9

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- „A“ document defining the general state of the art which is not considered to be of particular relevance
- „E“ earlier application or patent but published on or after the international filing date
- „L“ document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- „O“ document referring to an oral disclosure, use, exhibition or other means
- „P“ document published prior to the international filing date but later than the priority date claimed

- „T“ later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- „X“ document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- „Y“ document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- „&“ document member of the same patent family

Date of the actual completion of the international search

12 January 2001 (12.01.2001)

Date of mailing of the international search report

26 February 2001 (26.02.2001)

Name and mailing address of the ISA/AT

Austrian Patent Office
Kohlmarkt 8-10; A-1014 Vienna

Facsimile No. 1/53424/535

Authorized officer

WOLF

Telephone No. 1/53424/436

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR 00/01227

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
JP	A2	5056774	09-03-1993	none		
WO	A1	9842661	01-10-1998	AU	A1	65160/98
				EP	A1	984928
				IL	A0	120531
WO	A2	9923996	20-05-1999	AU	A1	11709/99
WO	A3	9923996	19-08-1999	EP	A2	1044009
				US	A	6046022
				AU	A1	46587/97
				BR	A	9712168
				EP	A1	932395
				HU	AB	9904650
				WO	A1	9814177
WO	A1	9937616	29-07-1999	AU	A1	41206/99
				GB	A0	9801398